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ORIGINAL PAPER

Gammaaminobutyric Acid A Receptor Alpha 3 Subunit is Overexpressed in Lung Cancer

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Abstract The identification of tumor-associated antigens, which are specifically expressed in cancer tissues, is very important for immunotherapy of lung cancer. We have combined the in silico screening and experimental verifying to identify genes that are differently expressed in cancers compared with their corresponding normal tissues. Using these methods, we have identified that GABRA3 gene was overexpressed in lung cancer and rarely expressed in other cancers. Furthermore, GABRA3 protein expression was significantly higher in the lower grade of lung cancer. It may compose functional GABA-gated channel with other subunits. This study demonstrated GABRA3 could be a potential biomarker for diagnosis of lung cancer, and GABAA receptors may play an important role in cancer differentiation.

Keywords Cancer · GABRA3 · Immunohistochemistry · In silico · RT-PCR

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Introduction

Lung cancer is the leading cause of cancer-related death in the world for both men and women. The 5 year survival rate for this cancer is low (16%) compared to other cancers [1]. Although we have known a lot of its biology and causes, the incidence of lung cancer is increasing, and improvements in prognosis are not clear [2]. But the mortality may be reduced from earlier detection of lung cancer. Currently, only a limited number of tumor markers for lung cancer are available. Identification of tumor-associated molecules is not only a crucial step in understanding their roles in a molecular mechanism in tumorigenesis, but also useful in clinical diagnostic and therapeutic applications.

High-thoughtput analysis has been very successful in identifying tumor-associated molecules to serve as diagnostic targets [3–5]. Recently, the sequence data in the public databases and the tools of bioinformatics have provided researchers excellent and time-saving approaches to identify genes that could be used as tumor diagnostic markers, prognostic indicators, and suitable targets for various forms of therapeutic intervention [6–8]. In the public database, expressed sequence tags (EST) are the most major sources to identify genes expressed in a variety of human tissues and in different pathological situations.

To identify the genes, which are differentially expressed in tumors compared with their corresponding normal tissues, a search was performed using the CGAP Expression Profiler based on EST data. And then the expression patterns of the candidate genes were evaluated by RT-PCR. Furthermore, immunohistochemistry was used to examine the tissue

expression patterns of the target gene on a panel of benign and tumor lung tissues. Using these methods, we found that the gammaaminobutyric acid (GABA) A receptor alpha 3 subunit (GABRA3) was overexpressed in lung cancers. The level of its protein expression was associated with the grade of the cancer. And it may compose functional GABAA receptors with other GABAA subunits.

Materials and Methods

Collection of Tissues

All samples of human cancerous tissues and paired noncancerous tissues (5 cm away from tumor) were obtained during surgical resection from the affiliated hospital of Weifang school of medicine. Patients agreed to collection of tissue samples with written consent. The resected tissue samples were immediately cut into small pieces, then, snap-frozen in liquid nitrogen until use. Paraffin sections of the tissues were kindly provided by Dr. Wenbo Huang (Affiliated Hospital of Weifang School of Medicine, China). Freshly dissected tissues were fixed in 10% buffered formalin and processed routinely for paraffin embedding. Paraffin-embedded specimens were sliced into 3 μm thick sections and mounted on silanized slides. All tumor tissue and paired noncancerous tissue samples were pathologically confirmed.

Cell Lines

Human cell lines used as followed: HepG2 (hepatocellular carcinoma) and L-02 (normal liver cell), MCF-7 and MDA-MB-231 (breast cancer), A375 (melanoma), CNE1 and CNE2 (nasopharyngeal carcinoma), HRT-18 (cancer of colon), U251 (glioma), HUVEC (human umbilical vein endothelial cell), HBEC (human bronchial epithelial cell) were maintained by our lab. All cell lines were cultured in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).

Bioinformatics

cDNA XProfiler Tool (http://cgap.nci.nih.gov/Tissues/xProfiler) based on EST libraries on the Cancer Genome Anatomy Project web site was used to identify genes expressed specifically in cancer tissues. All EST libraries were selected with the exception of the libraries from embryonic tissues, germ cells, testis and whole body or the libraries which were uncharacterized. Microdissected tissues and cell lines were selected as the tissue preparation. Pool A was from normal tissues and Pool B from cancer tissues. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) on the NCBI web site was used for Sequence Similarity Searching.

The UniGene and SAGE database were accessed to establish an electronic expression profile (E-Northern) for each of the hits to facilitate tumor- and organ-selective gene discovery.

RNA Isolation and cDNA Preparation

Total RNA was extracted from liquid-nitrogen-frozen tissue samples by homogenization in Trizol reagent (Invitrogen), and First-strand cDNAs were synthesized from 2 μg of DNase I-treated total RNA using oligo(dT) primer with SuperscriptTM II reverse transcriptase (Invitrogen) for 60 min at 42°C.

Relative Quantitative PCR

Gene-specific PCR primers were used to amplify cDNA fragments of GABAA-α1-6, β1-3, γ1-3, δ, ε, θ, π and GAPDH. The sequences of the GABAA receptor oligonucleotide sense and antisense primers are provided in Table 1. The primer designed for GAPDH is described as the following: GAPDH of 580 bp, 5′- AATCCCATCACCATC TTCCA -3′, (sense) and 5′- CCTGCTTCACCACCTTC TTG -3′ (antisense). 1 μl of each cDNA product was amplified in a mixture containing 5 pmol primers, 200 μM dNTPs, and 1 unit Taq DNA polymerase with reaction buffer in a final volume of 20 μl. PCR was performed using the following parameters: initial denaturation at 95°C for 1 min; 20, 25, 30, and 35 cycles of 30 sec at 95°C; 30 sec at 60°C; and 1 min 30 sec at 68°C; followed by a final 10 min extension at 68°C. Reaction products were separated on 1.5% agarose gels containing ethidium bromide and the level of amplification was determined using a Phosphor Imager. There is no obvious PCR product detected when using 20 cycles and 25 cycles, and there is no significant difference among different cell lines and tissues when using 35 cycles. So the 30 cycles should be the best parameter for detecting and evaluating the amounts of PCR products. The relative expression was measured as a ratio of GABAA receptor subunit expression to GAPDH expression by using the products of 30 cycles.

Immunohistochemistry

Sections were baked for 40 min at 70°C followed by dewaxing and hydration through a graded ethanol series and washed with PBS. To recover antigens the slides were treated in a dampness cooker with a steam solution of 10 mmol/L citrate pH 6.0 at 95–100°C for 20 min. Endogenous peroxidases was blocked with 3% H_2O_2 in methanol for 30 min, and following that, non-specific sites

Table 1 Primers for GABAA receptor subunits

Subunit	Primer	Product size (bp)
α1		902
Sense	5′-TCGTCACCAGTTTCGGACC-3′	
Antisense	5′-GGTTGCTGTTGGAGCGTAA-3′	
α2		722
Sense	5′-TTCACAATGGGAAGAAATCAGTAG-3′	
Antisense	5′-TGCATAAGCGTTGTTCTGTATCA-3′	
α3		561
Sense	5′-TCGGTCTCTCCAAGTTTGTGC-3′	
Antisense	5′-TTCCGTTGTCCACCAATCTGA-3′	
α4		750
Sense	5′-TGAAATTCGGGAGTTATGCCTATC-3′	
Antisense	5′-GGCTGAATGGGTTTGGACTG-3′	
α5		826
Sense	5′-CACCATGCGCTTGACCATCTCT-3′	
Antisense	5′-GCCGAACAAGACTGGGAATA-3′	
α6		764
Sense	5′-TGAGGCTTACCATCAATGCTGA-3′	
Antisense	5′-GACAGGTGTTGATTGTAAGATGGG-3′	
β1		603
Sense	5′-GTTCTCTATGGACTCCGAATCACA-3′	
Antisense	5′-ATTGGCACTCTGGTCTTGTTTG-3′	
β2		640
Sense	5′-AGCTTAAGAGAAACATTGGCTACT-3′	
Antisense	5′-CGATCTATGGCATTCACATCA-3′	
β3		633
Sense	5′-AGTGCTGTATGGGCTCAGAATCAC-3′	
Antisense	5′-CCCGGTTGCTTTCGCTCTT-3′	
γ1		262
Sense	5′-GTGTTTTGCAGCCTTGATGG-3′	
Antisense	5′-TGGCAATGCGTATGTGTATCCT-3′	
γ2		605
Sense	5′-AAGTCCTCCGATTGAACAGCAACA-3′	
Antisense	5′-CGCTGTGACATAGGAGACCTT-3′	
γ3		767
Sense	5′-ACACTCCTGCCCGCTGATT-3′	
Antisense	5′-TGTCTATGTGAATACGCCCTTTCC-3′	
Δ		654
Sense	5′-TCACCATCACCAGCTACCACTTCA-3′	
Antisense	5′-GGGCGTAAATGTCAATGGTGTC-3′	
E		632
Sense	5′-GCAGGCGGTTTGGCTATGT-3′	
Antisense	5′-CGAGTAGTTATCCAGGCGGTAG-3′	
Θ		465
Sense	5′-TCGAGTTCTCCTCTGCTGTG -3′	
Antisense	5′-TATGCAGATCCAGGGACAA-3′	
π		250
Sense	5′-CGTCGAGGTCGGCAGAAGT-3′	
Antisense	5′-GCGGGCATCCAGAGTGAAG-3′	

were bound with 5% BSA for 20 min. Then, the samples were incubated with rabbit anti-human polyclone antibody against GABRA3 (1:200 dilution, Chemicon) at 4°C over night and then further developed using an anti-rabbit IgG ABC kit (Boster) and DAB kit (Boster). The procedures were performed according to the manufacturer's instructions. Staining intensity was graded as weak, 1; moderate, 2; or strong, 3. The stained area was classified as follows: no staining, 0; ≤10% of all cells stained as viewed by microscopy, 1; 11% to 50%, 2; 51% to 75%, 3; >75%, 4. An average immunoreactive score was calculated by multiplying the staining intensity by the area of staining. A total score of 3 or more was defined as positive expression and a score of less than three as negative. 0~2, (−); 3~4, (+); 6~8, (++); 9~12, (+++).

Statistical Analyses

Chi-square test was performed to compare the GABRA3 differential expression levels in different categories of lung cancers.

Results

Results in Silico Screening and in E-Northern

In our analysis, 39 known genes unique in pool B were first identified, and after secondary analysis using SAGE data, we focused on one unigene cluster Hs.632390 which matched to the known gene GABRA3 (Hs.123024). By E-Northern, the expression map had been demonstrated to provide an evidence that the gene GABRA3 maybe a cancer-associated gene (Table 2). It only expressed in the tissues below, none of the others.

Expression Profile of GABRA3

The mRNA expression profile of GABRA3 was determined by RT-PCR. A series of tissues and cell lines were examined, including normal tissues, tumor and normal cell lines, lung cancer and corresponding adjacent noncancerous tissues, esophageal carcinoma, stomach cancer, colon cancer, rectal carcinoma, breast cancer, bladder carcinoma, renal carcinoma, endometrial cancer, ovarian cancer and glioma tissues.

Expression in Normal Tissues

For the detection of mRNA expression of GABRA3, there were 20 different normal tissues of lung, brain, cervix, endometrium, kidney, tonsil, muscle, colon, stomach, breast, esophagus, liver, bone, ovary, skin, thyroid, placenta, lymph node, bladder and prostate being tested by RT-PCR, GABRA3 was strongly expressed in brain and placenta, and weakly detected in endometrium and prostate, but undetectable in other tissues (Fig. 1). Relative quantification

Table 2 Expression map of Hs.123024 by e-northern

Tissue	EST Data		SAGE Data		EST Data			SAGE Data		
	Normal	Cancer	Normal	Cancer	Normal	Cancer	P	Normal	Cancer	P
ALL TISSUES					47 / 3384626	13 / 2592199	0.00	1 / 7867893	1 / 11929783	0.45
brain					23 / 275764	3 / 206552	0.00	1 / 729526	0 / 4805583	0.27
cartilage			--		0 / 15123	0 / 37251	--	--	1 / 778035	--
cerebellum					1 / 85643	0 / 0	--	0 / 90885	0 / 1495938	--
cerebrum			--	--	14 / 202152	0 / 3410	0.43	--	--	--
head and neck			--	--	0 / 42869	2 / 102132	0.34	--	--	--
mammary gland					0 / 55170	1 / 115666	0.41	0 / 509556	0 / 1717147	--
nervous			--	--	9 / 230741	0 / 0	--	--	--	--
testis			--		0 / 146512	2 / 121229	0.24	--	0 / 66352	--
uncharacterized tissue					0 / 162762	4 / 345009	0.21	0 / 21833	0 / 51729	--
uterus				--	0 / 38602	1 / 140659	0.43	0 / 21991	--	--

of gene expression revealed that the expression level of GABRA3 mRNA in the brain was two-fold higher than that in placenta and five-fold than endometrium and ten-fold than prostate.

Expression in Cell Lines

A panel of 11 cell lines (hepatocellular carcinoma or normal liver cell: HepG2 and L-02; breast cancer: MCF-7 and MDA-MB-231; melanoma: A375; nasopharyngeal carcinoma: CNE1 and CNE2; cancer of colon: HRT-18; glioma: U251; human umbilical vein endothelial cell: HUVEC; human bronchial epithelial cell: HBEC) were tested. GABRA3 was highly expressed in hepatocellular carcinoma line HepG2, and weakly detected in glioma cell line U251 and breast cancer cell lines MCF-7 and MDA-MB-231 (Fig. 2).

Expression in Lung Cancers, Adjacent Noncancerous Lung Tissues and Other Cancer Tissues

GABRA3 mRNA transcripts were analysed for their expression in the samples of lung cancer tissues and other cancer

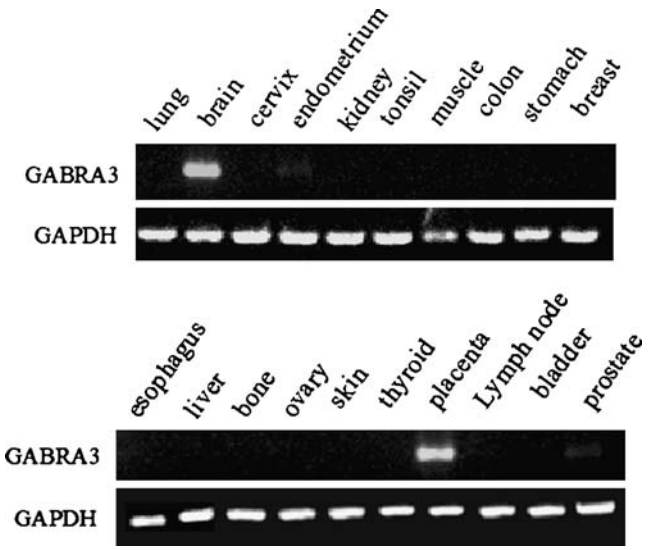

Fig. 1 Expression of GABRA3 in different normal tissues by RT-PCR. GABRA3 was strongly expressed in brain and placenta tissues, weakly expressed in endometrium and prostate tissues. Expression of GAPDH serves as the quantitative control

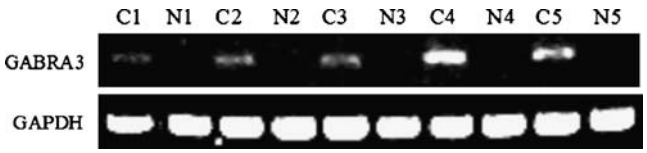

Fig. 3 Expression of GABRA3 in lung cancerous tissues (C1-5) and adjacent tissues of lung cancers (N1-5) by RT-PCR. GABRA3 was only detected in lung cancerous tissues, not in adjacent tissues. Expression of GAPDH serves as the quantitative control

tissues. It was significantly expressed in one out of 18 lung cancer samples (5.56%) and weakly expressed 11 out of 18 (61.11%), but not expressed in corresponding adjacent noncancerous lung tissues (Fig. 3). For GABRA3 was only detected in lung cancer tissues, not in adjacent noncancerous lung tissues, it should be also reliabe to obtain the significant difference by using semiquantitive PCR comparing to real-time PCR analysis. More importantly, the expression of GABRA3 in other cancer types was both rare and less abundant but with the exception of glioma and endometrial cancer (Table 3). Although it was significantly expressed in glioma and endometrial cancer, GABRA3 was also detected in normal brain and endometrium tissues.

Immunohistochemistry

To further investigate the tissue expression pattern of GABRA3 gene in lung tissues, we performed immuno-

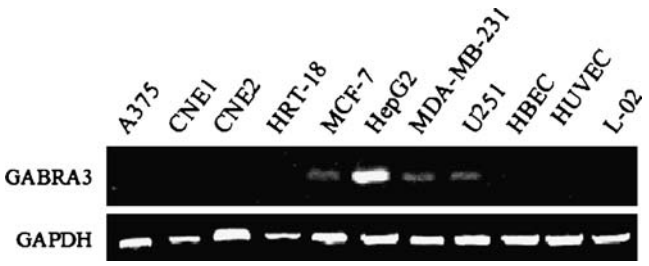

Fig. 2 Expression of GABRA3 in different cell lines by RT-PCR. GABRA3 was stongly expressed in HepG2, weakly expressed in MCF-7, MDA-MB-231 and U251. Expression of GAPDH serves as the quantitative control

histochemistry analyses on the specimens of squamous carcinoma (SC), adenocarcinoma (AC) and corresponding normal tissues. For each category, one set of representative staining was shown in Fig. 4. The experimental results demonstrated cytoplasmic and membrane staining in SC and AC cells, but not in normal tissue cells. Table 4 further showed the correlation between GABRA3 protein expression and clinical-pathologic variables in lung cancers. GABRA3 protein expression was significantly higher in lower grade of lung cancer ($p<0.05$). These results demonstrated that GABRA3 gene could be used potentially as a biomarker for diagnosis of lung cancer.

GABAA Receptor Subunits mRNA Expression in Lung Cancers

To determine whether GABRA3 composes functional GABAA receptors with other GABAA subunits in lung cancers, we documented GABAA receptor subunits mRNA expression in 12 lung cancers which expressed GABRA3. The result of semiquantitative PCR on a scale of + (limited expression) to +++ (highly expressed) are provided in Table 5. In these samples GABAA-β3 (8/12), −δ (6/12), −π (12/12), and −θ (12/12) subunits are also expressed.

Table 3 Expression of GABRA3 in different cancerous tissues

Cancer	Total	−	+	++	+++	Positive (rate, %)
Esophageal carcinoma	2	2	0	0	0	0
Stomach cancer	9	9	0	0	0	0
Colon cancer	7	7	0	0	0	0
Rectal carcinoma	4	4	0	0	0	0
Breast cancer	7	7	0	0	0	0
Bladder carcinoma	4	4	0	0	0	0
Renal carcinoma	2	2	0	0	0	0
Endometrial cancer	11	0	0	4	7	100
Ovarian cancer	4	4	0	0	0	0
Glioma	3	0	2	1	3	100

−: no expression; +: limited expression; ++: moderate expression; +++: high expression

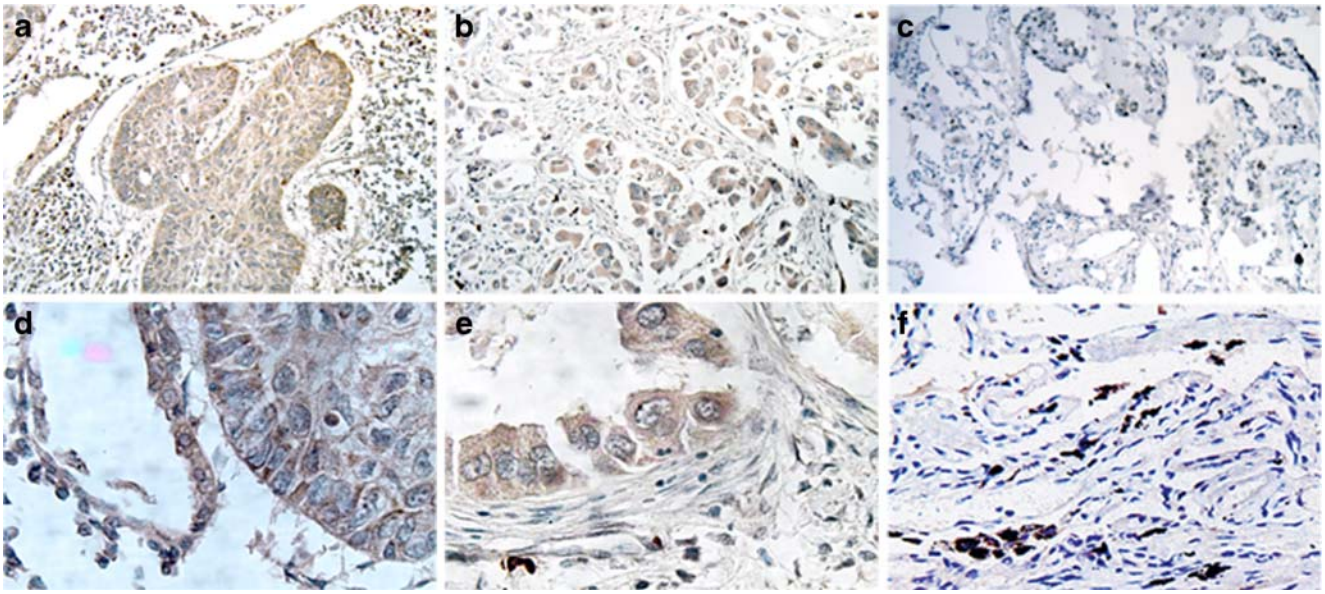

Fig. 4 Immunohistochemistry results of GABRA3. A. AC of lung, 100×, B. SC of lung, 100×, C. adjacent tissues of lung tumor, 100×, D. AC of lung, 400×, E. SC of lung, 400×, F. adjacent tissues of lung tumor, 400×. Cytoplasmic and membrane were stained in SC and AC cells, but not in non-tumor lung tissues

Discussion

The etiological factors of lung cancer are not clear now, but recent researches showed that the tumor progression is germane to somatic genetic changes and is reflected in phenotypic changes such as altered gene expression profiles. Discovery of tumor-associated genes gives us chances to fight against cancers. Owing to the databases and data-mining tools on the websites, we can capture information on tens of thousands genes simultaneously, and some genes might be related to tumors. In this study, our approach gives a quick search for genes which may be useful as improved biomarkers for diagnosis or as targets for developing novel treatment methods. But the EST-based strategy has many limitations some of which have been discussed [9–11]. The genes searched based on the EST libraries may not be closely related to tumors due to the poor sequencing depth of libraries and differences in library sizes. Their expression profiles need to be experimentally tested by RT-PCR. Another related problem is that the identified variations in transcript expression may not correlate with similar variations in the encoded protein, so it is necessary to experimentally test the computer-based predictions either by western blotting or immunohistochemistry.

In this study, we discovered the overexpression of GABRA3 in more than half of the lung cancers and found that GABRA3 was moderately expressed in few normal organs and other tumors. These results implicate that GABRA3 is a good molecular target for the development of novel lung cancer therapies with a minimal risk of side effects, with regards to its expression pattern. GABRA3 was overexpressed in lung tumors from all histopathological grades of SC and AC. The expression level of GABRA3 was highest in the poorly-differentiated tumors. This suggests

Table 4 Correlation between GABRA3 protein expression and clinical-pathologic variables in lung cancers

Variable	*n*	−/+	++/+++	*P* value
Gender				0.593
Male	36	20	16	
Female	24	15	9	
Age				0.452
<60	25	16	9	
≥60	35	19	16	
Smoking history				0.526
Yes	38	21	17	
No	22	14	8	
Histotype				0.432
SC	30	19	11	
AC	30	16	14	
Grade				0.000
I	16	15	1	
II	30	17	13	
III	14	3	11	
Lymph node status				0.526
N+	38	21	17	
N–	22	14	8	

"*n*" represents total sample number for each category, Chi-square test was performed to compare the GABRA3 differential expression levels in different categories of lung cancers. Grade I: well differentiation, grade II: moderately differentiation, grade III: poorly differentiation

Table 5 GABAA receptor subunits mRNA expression in 12 lung cancer tissues

Subunit	Set											
	1	2	3	4	5	6	7	8	9	10	11	12
α1												
α2												
α3	++	+	+	+	+	+	+	+	+	+	+	+
α4												
α5												
α6												
β1												
β2												
β3	+		+			++		+	+	+	++	+
γ1												
γ2												
γ3												
δ	+		+	+			+		+		+	
π	+	++	+	+	+	+	+	+	++	+	+	+
θ	++	++	+	++	+	+	+	++	++	+	++	+
ε												

+: limited expression; ++: moderate expression; +++: high expression

that increased transcription of GABRA3 gene is a sustained event in the tumorigenic process and it could become a new marker for lung cancers.

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain. It mediates its effects via the specific interaction with the integral membrane proteins, the GABA receptors which are grouped on the basis of their pharmacology under three major classes of receptors: GABAA, GABAB, and GABAC receptors. GABRA3 is a subunit of the GABAA receptors that may associate with other GABAA receptor subunits to form a functional chloride channel. GABAA receptors are pentameric complexes, usually composed of two α subunits, two β subunits, and either a γ2 subunit or a low-abundance subunit, such as δ. In this study, GABAA-β3, -δ, −π, and −θ subunits were also detected in lung cancers which expressed GABRA3. This finding suggests that these subunits may compose a functional GABA-gated channel. Regarding the expression of GABRA3, that it may form a functional GABAA receptor channel with other subunits has not been previously described, and the significance of this finding has yet to be elucidated.

Previous researches have found that the GABAA receptor subunit expression correlates with the state of cell differentiation [12]. This finding is similar to our results. Chloride channels regulated by different mechanisms have been associated with several types of cancers, e.g. the volume-sensitive chloride channels are expressed in colon adenocarcinoma, transformed tracheal epithelium, and cervical cancer [13, 14]. Volume-activated plasma membrane chloride channel plays an important role in control of cell proliferation in nasopharyngeal carcinoma. When the channel was blocked, arrest of cell growth in G0/G1 phase was tested [15]. The same result was also found in cervical cancer [16]. Membrane ion channels also play a major role in tumor cell migration because of the changes in shape and volume when the cells migrate. Cell migration was increased when treated with hypotonic solutions, which was shown to activate Cl^- current. Hypertonic treatments, which suppressed Cl^- current, inhibited cell migration [17]. These studies demonstrated a role of chloride channel in carcinogenesis and migration.

In previous researches, GABA receptors were found overexpressed in pancreatic cancer, breast cancer, etc [18–20]. Giammarco et al reported that GABA decreases biliary cancer proliferation and reduces the metastatic potential of cholangiocarcinoma [21]. And Joseph et al reported that GABA could inhibit the norepinephrine-induced migration of colon cancer [22]. These results lend us to the investigation of the possible use of GABA and GABA receptor agonists for the chemoprevention of cancers. On the other hand, there is a report showed that GABA is a promoting factor of prostate cancer metastasis and invasion through the regulation of metalloproteinase production [23]. Akio et al found that GABA promoted the proliferation of GABRP-expressing PDAC cells, but not GABRP-negative cells, and GABAA receptor antagonists inhibited this growth-promoting effect by GABA [19]. Although some of the results above are conflicted, we could find that GABA receptors play an important role in cancer proliferation, differentiation and

migration. The detection of GABAA receptors in lung cancers lend us to take more investigations of their roles in cancers.

Although the mechanism of GABRA3 and GABA in lung cancer is still unclear, further investigation should be taken to understand the functional properties of them, GABRA3 may serve as a molecular marker and target for diagnostic and therapeutic applications for lung cancer.

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